

Nonylphenol-induced hepatotoxicity in the freshwater fish, *Oreochromis mossambicus*

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Abstract- Adult freshwater fish, *Oreochromis mossambicus* was exposed to nonylphenol at sublethal concentration (0.15 mg/ L) for 24 h, 96 h and 7 days. Nonylphenol significantly decreased the weights of liver and also hepato-somatic index at the end of 7 days, however no such changes were observed at 24 and 96 h of treatment. Decreased weight may be due to necrosis or atrophy of hepatocytes, which is evidenced by the histopathological observations. The activities of antioxidant enzymes decreased after 24 h and 96 h whereas at 7 days of exposure the activities of antioxidant enzymes begin to increase and the levels of hydrogen peroxide and lipid peroxidation decreased to normal level like that of control groups. In general, repeated doses of nonylphenol for 7 days, may induce a defensive response in *Oreochromis* meanwhile, acute exposure caused inhibition of antioxidant activities. The present study also showed a significant decrease in the marker enzyme, alkaline phosphatase after 96 h indicating the decreased state of inter and intracellular membrane transport and the activity of enzyme increased to normal level at 7 days. In histopathological observation, the control liver exhibited a normal architecture with hepatocytes presenting a homogenous cytoplasm and a large central or subcentral spherical nucleus. In the treated group an increase in cytoplasmic vacuolization, necrosis and decrease in the number of hepatocyte nucleus was observed. This could be possibly owing to the toxicity of nonylphenol and also due to the generation of reactive oxygen species in fish hepatocytes.

Index Terms- Nonylphenol, liver, oxidative stress, alkaline phosphatase, antioxidant

I. INTRODUCTION

Animals when exposed to the environmental pollutants have been recognized to induce alteration of different magnitudes in the physiological and biochemical status of the being. Therefore, to assess the environmental impact of those compounds on animals, certain biomarker parameters are used that provide quantitative response as well as valuable information on the acute/chronic adverse effects caused by those pollutants. However, the changes in biochemical, physiological or histopathological alterations are considered as an adaptive mechanism which allows the fish to cope with real or perceived stressors so that the normal homeostatic state could be maintained¹. Fishes exposed to toxicants are expected to undergo stress, which are a state of re-established homeostasis and a complex suite of maladaptive responses. Under stress, some of the biochemical responses may be compromised, becoming detrimental to the fish's health and well being, at this point the fish is termed as distressed. In the present study, the response of

fish to one of the environmental pollutants nonylphenol was evaluated on hepatic antioxidant status.

Nonylphenol is widely used as a chemical intermediate in the production of nonylphenol ethoxylates and other compounds. It is an important class of non-ionic surfactants widely used in industrial applications such as paper and textile manufacture, paints, resins, coatings, adhesives, plastic additive in modified polystyrene and polyvinyl chloride and also as industrial cleaners. An important source of nonylphenol in the environment is unreacted nonylphenol in plastic, which may result in direct human exposures when the chemical leaches out of plastic in close contact with foods. Ever since nonylphenol was first synthesized in 1940, its use and production have been increasing almost exponentially. The annual production of nonylphenol reached 154,200 tons in the USA, 73,500 tons in Europe, 16,500 tons in Japan and 16,000 tons in China. However, many other countries including India use and produce nonylphenolic compounds in large amounts and no action has been taken by the government to reduce or eliminate their usage.

Nonylphenol is moderately lipophilic and in the higher organisms the bioconcentration levels may include biomagnification through the food chain, and also uptake of sediment containing higher levels than in the water thus accumulate in both freshwater and marine organisms². Nonylphenol was found to mimic the natural hormone 17 β -estradiol by competing for the binding site of the estrogen receptor due to their structural similarity³. Nonylphenol has been shown to reduce the epididymal sperm count along with induction of an oxidative stress in the epididymal sperm of rat⁴. However, co-administration of vitamin E prevented nonylphenol-induced oxidative stress in testis of rat⁵.

Nonylphenol at sublethal concentration caused genetic damage in freshwater fish, *Oreochromis mossambicus* as evidenced by micronucleus test and Salmonella mutagenicity test⁶. Nonylphenol also have damaging effects on important physiological process and reproductive tissues, induce apoptosis in many organisms, affects sperm motility, gene expression of pituitary hormones and oxidative damages of organs. It also interfere with synthesis, secretion, transport, metabolism, binding action or elimination of natural blood-borne hormones that are present in the body and are responsible for homeostasis, reproduction, and developmental process. The study aimed to determine duration-dependent effects of nonylphenol on the hepatic antioxidant status of the freshwater fish, *Oreochromis mossambicus*. It also examined the extent to which the nonylphenol toxicity pave mode to the histopathological alterations in fish hepatocytes.

II. METHODS

Fresh water fish, *Oreochromis mossambicus* weighing 6.5 ± 2 g and length 7.5 ± 1 cm were collected from Kaloos fish farm, Malappuram District, Kerala, India. Fishes were acclimatized to the laboratory conditions for four weeks with constant supply of water and good lighting system. During the period of acclimatization, fishes were fed everyday with standard fish pellets. Bath was changed every 24 hour, which was dechlorinated, respectively.

The physico-chemical features of the tap water were estimated as per APHA⁷. Water temperature in the test ranged from $28 \pm 2^\circ\text{C}$ during the experiment, oxygen saturation of water ranged between 70 and 100 %, pH is 6.5 to 7.5 which were monitored using a standardized procedures. The LC_{50} values in the respective time intervals were determined by probit analysis, with a confident limit of 5 % level⁸, which was 1.5 mg/ L. One-tenth of the dosage (0.15 mg/ L) nonylphenol was chosen to represent sub-lethal concentration.

The specimens were not fed a day prior to and during toxicity tests to reduce faecal and excess food contaminating the test solution. Ten specimens were placed in each tub and were maintained in each test and control groups, they were then aerated using tubed motorized pumps. Monofilament netting was used to cover the tanks to prevent the specimens from jumping out of test solutions. The behaviour of specimens was observed and death was also recorded throughout the study.

Treatments:

There were four groups, three tanks each with toxicant doses maintained for 24 h, 96 h and 7 days, respectively and a tank with control fishes. Single dose with different durations were used in present study. Ten fish specimens were used for every test and also in control groups. The first groups of fishes were maintained in toxicant-free water and were used as control and the second group was treated with nonylphenol at 0.15 mg/ L for 24 h. The third group was treated with nonylphenol at 0.15 mg/ L for 96 h and fourth group was treated with nonylphenol at 0.15 mg/L for 7 days. Biochemical estimation of liver was performed at the end of every treatment and the histopathology was also done at the end of experiments, maintaining the control group.

Tissue processing:

A 1% (w/ v) homogenate of liver was prepared in ice-cold normal saline with the help of a motor-driven glass Teflon homogenizer on crushed ice for a minute. The homogenate was centrifuged at 8000 g for 15 min at 4°C to obtain the supernatant, which was then used for the analyses.

Biochemical analysis:

Total protein concentration in the tissue was estimated by the method of Lowry et al.⁹. The levels of lipid peroxidation were measured via the thiobarbituric acid color reaction for malondialdehyde (MDA) at 535 nm, according to the method of Ohkawa et al.¹⁰. Hydrogen peroxide generation was assayed by the method of Pick and Keisari¹¹. Superoxide dismutase (EC 1.15.1.1) was assayed by the method of Marklund and

Marklund¹². Catalase (EC. 1.11.1.6) was assayed by the method of Claiborne¹³. The activity of alkaline phosphatase (EC.3.1.3.1) was assayed by the method of Bessey et al.¹⁴.

Histopathology:

Liver tissue collected by sacrificing the fish was fixed in 10 % buffered formalin for 24 hours. Tissue was dehydrated in ascending grades of alcohol and was cleared in xylene until they became translucent. It was then transferred to molten paraffin wax for 1 hour to remove xylene completely and then impregnated with wax. Then the blocks were cut in a rotary microtome to prepare sections of thickness 4 to 6 microns. The sections were stained with haematoxylin and eosin and mounted in DPx. The structural alteration was observed under light microscope in the sections of liver of fish and was compared with those of control tissues. Photomicrographs were taken using Cannon shot camera fitted to the Carl Zeiss Axioscope 2 Plus Trinocular Research Microscope.

Statistical analyses:

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $p < 0.05$ against control group. Data are presented as mean \pm SD for ten animals per group. All biochemical estimations were carried out in duplicate.

III. RESULTS

Nonylphenol at the sub lethal concentration of 0.15 mg/ L at 24 h and 96 h showed no remarkable changes in the weights of liver and hepato-somatic index, but a significant decrease was observed after 7 days of treatment (Table 1). The activities of superoxide dismutase and catalase decreased significantly at 24 h and 96 h, however after 7 days of treatment the activities of superoxide dismutase and catalase significantly ($p < 0.05$) increased when compared with control groups (Table 2).

The level of hydrogen peroxide and lipid peroxidation increased significantly ($p < 0.05$) at 24 h, 96 h of nonylphenol treatment in liver of fishes and no significant changes were observed after 7 days of treatment (Table 2). In liver tissue the activity of alkaline phosphatase showed a significant ($p < 0.05$) decrease after 96 h of exposure whereas no changes were observed at 24 h and 7 days of nonylphenol treatment than that of control group (Table 2).

In the control group, the liver exhibited a normal architecture and there were no pathological abnormalities, with hepatocytes presenting a homogenous cytoplasm, and a large central or subcentral spherical nucleus (Figure 1A). The hepatic parenchyma of fish exposed to nonylphenol after 7 days showed a tremendous decrease in number of hepatocyte nucleus (Figure 1B). Additionally, fishes exposed to nonylphenol also showed hepatocellular necrosis (Figure 1C) and an increase in cytoplasmic vacuolization (Figure 1D).

Table 1 Effect of nonylphenol on the body weight and tissue weights of the fresh water fish, *Oreochromis mossambicus*

| Weights | Nonylphenol (0.15mg/ L) | | | |
|--------------------------|-------------------------|-------------|-------------|---------------|
| | Control | 24 h | 96 h | 7 days |
| Liver (mg) | 113 ± 0.12 | 101 ± 0.29 | 107 ± 0.09 | 82 ± 0.18* |
| Hepato-somatic index (g) | 1.45 ± 0.54 | 1.24 ± 0.39 | 1.28 ± 0.81 | 0.987 ± 0.22* |

Data are expressed as Mean ± SD for 10 animals/ group. Asterisks (*) denote the p value set significant at 0.05 level of significance against the control group.

Table 2 Effect of nonylphenol on the biochemical parameters of the fresh water fish, *Oreochromis mossambicus*

| Parameters | Nonylphenol (0.15mg/ L) | | | |
|-----------------------------------|-------------------------|----------------|----------------|--------------|
| | Control | 24 h | 96 h | 7 days |
| Superoxide dismutase ^a | 0.376 ± 0.091 | 0.054 ± 0.022* | 0.071 ± 0.007* | 0.5 ± 0.158 |
| Catalase ^b | 13.168 ± 2.01 | 0.385 ± 0.233* | 0.576 ± 0.365* | 13.63 ± 4.30 |
| Hydrogen peroxide ^c | 7.32 ± 3.44 | 13.34 ± 3.52* | 17.22 ± 1.53* | 5.90 ± 1.84 |
| Lipid peroxidation ^d | 18.17 ± 2.76 | 66.71 ± 17.9* | 78.78 ± 5.33* | 15.60 ± 3.06 |
| Alkaline phosphatase ^e | 18.1 ± 0.34 | 16.41 ± 0.87 | 10.17 ± 0.38* | 14.79 ± 0.47 |

Data are expressed as Mean ± SD for 10 animals per group. Asterisks (*) denote the p value set significant at 0.05 level of significance against the control group.

- a nmol pyrogallol oxidised/ min/ mg protein
- b µmole of hydrogen peroxide consumed/ min/ mg protein
- c nmole hydrogen peroxide generated/ min/ mg protein
- d nmol of malondialdehyde produced/ min/ mg protein
- e µmole of p-nitrophenol liberated/ 30 min/ mg protein

FIGURE 1A Control liver showing normal architecture. No pathological abnormalities, hepatocytes showing a homogenous cytoplasm, and a large central or subcentral spherical nucleus (40x magnification)

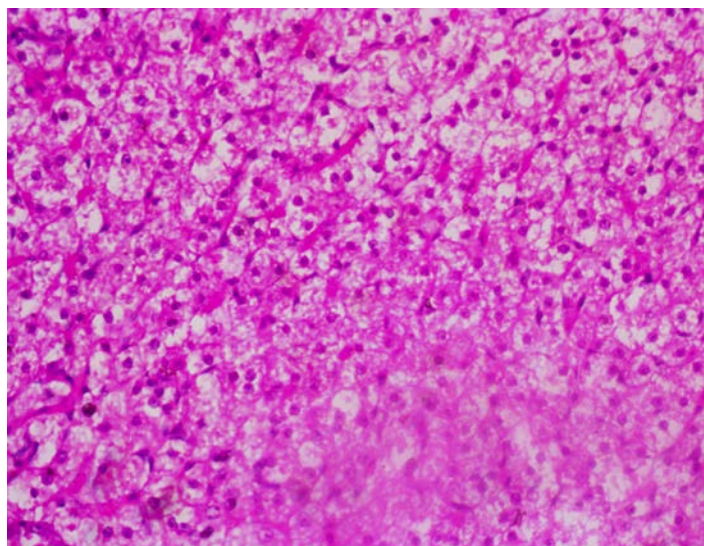


FIGURE 1B Hepatic parenchyma of fish exposed to nonylphenol after 7 days showed a complete disappearance of nucleus (40x magnification)

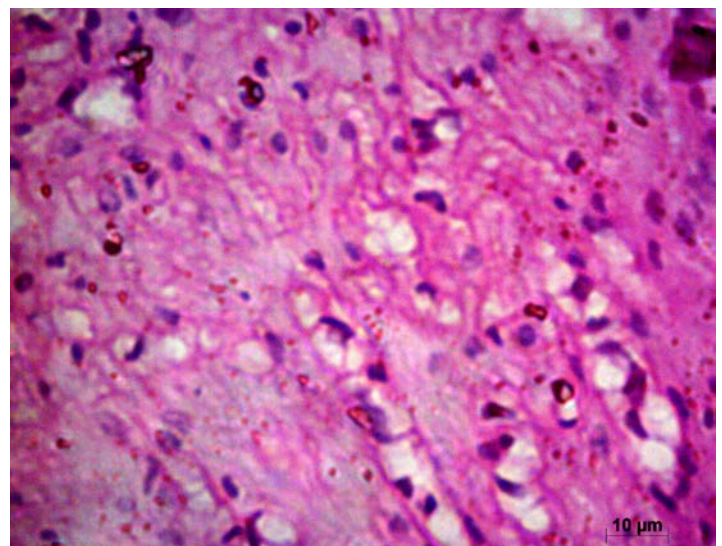


FIGURE 1C Exposure to nonylphenol for 7 days showed hepatocellular necrosis (40x magnification)

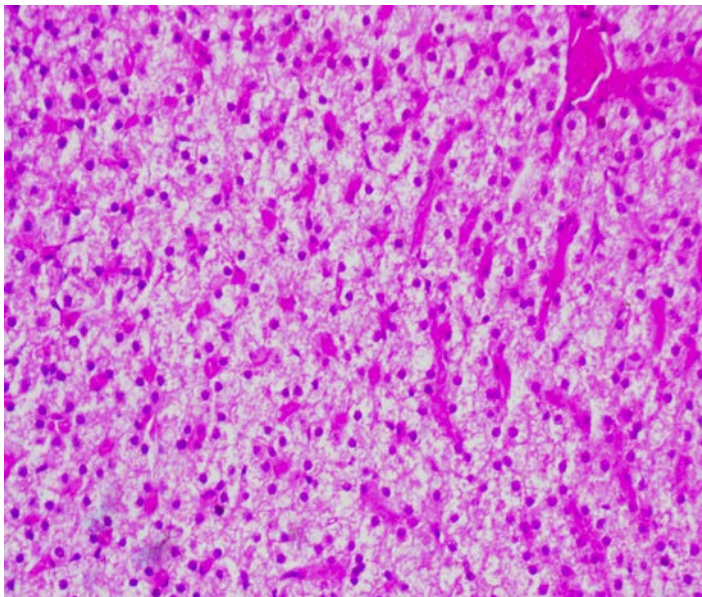
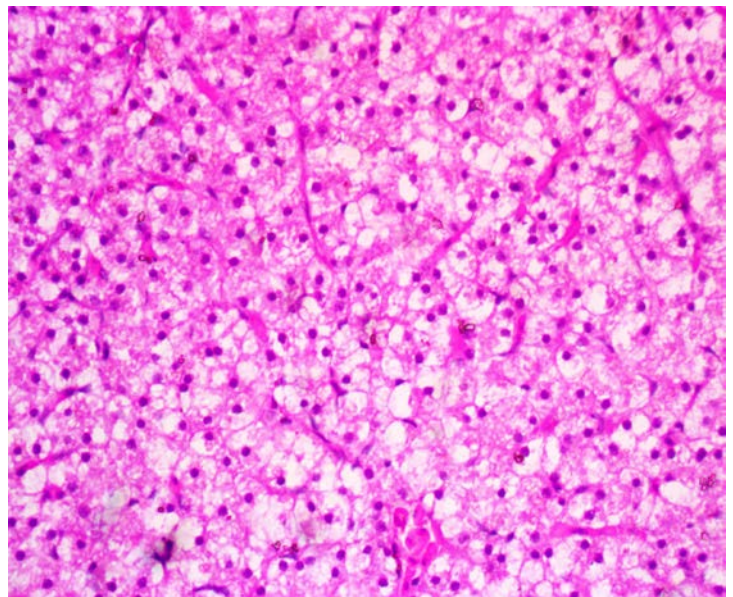


FIGURE 1D Exposure to nonylphenol for 7 days showed increase of cytoplasmic vacuolization in hepatocytes (40x magnification)



IV. DISCUSSIONS

Nonylphenol are ubiquitous and they are known to resist degradation and bioaccumulate, as it also possesses ability to produce physiological effects that made a subject of considerable recent attention. Several studies have reported that nonylphenol is directly toxic to fish where aquatic communities are exposed indirectly through runoff from land treated with sewage sludge. Even low concentrations of nonylphenol lead to estrogenic effects that might have long-term consequences, such as disrupting population dynamics or interactions within the aquatic communities. Many studies have reported the effects of nonylphenol on reproduction and development in vertebrates including mammals besides their interactions with the endocrine system, however, little is known about the effect of nonylphenol on the antioxidant status in fishes. However one of our previous reports stated that nonylphenol induced oxidative stress in gill of fish¹⁵. Therefore, the goal of the present study was to scrutinize the effect of nonylphenol on the generation of reactive oxygen species (ROS) in hepatocyte of the fresh water fish, *Oreochromis mossambicus*.

Liver is considered as the main and important detoxifying organ and is essential for both the metabolism and the excretion of toxic substances in the body; and several categories of hepatocellular pathology are now regarded as reliable biomarkers of toxic injury and are representative of biological endpoints of contaminant exposure¹⁶. The effects of environmental pollutants may be assessed based on the changes in the health status of humans or ecosystems, or selected species. Health is a qualitative endpoint but must be expressed in a measurable way. For example, endpoints such as the production of reactive oxygen species in fish are used in the present study to quantify the effect of the toxicant, nonylphenol on the freshwater fish, *Oreochromis mossambicus*. As additional evidence the histopathological changes were also analyzed. In the present

study the decreased weights of liver and also the hepato-somatic index could be possibly due to necrosis or atrophy of hepatocytes which is evidenced by the histopathological observations.

Environmental pollutants generally cause oxidative stress by an increase in peroxidative processes within cells. Some of the important cell components such as proteins and DNA are damaged due to the potent oxidants as hydroxyl radicals that are being produced in electron transfer reactions¹⁷. Lipid peroxidation has often been used as a biomarker of environmental stress, reflecting damage to cell membranes from free radicals and is an important feature in cellular injury¹⁸. The extent of damage caused by oxyradical production is dependent on antioxidant defences, which include antioxidant enzymes and free radical scavengers. Therefore, antioxidant enzymes are some of the most common biomarkers used in environmental monitoring. These enzymes usually respond rapidly and sensitively to biologically active pollutants¹⁹. In the present study the activities of antioxidant enzymes decreased after 24 h and 96 h in the hepatocytes of nonylphenol-treated fishes whereas at 7 days of exposure the activities of antioxidant enzymes begin to increase and the levels of hydrogen peroxide and lipid peroxidation decreased to normal level like that of control groups. In general, repeated doses of nonylphenol for 7 days could have induced a defensive response in *Oreochromis* meanwhile acute exposure caused inhibition of antioxidant activities and elevation of reactive oxygen species in fish hepatocytes.

The present study also showed a significant decrease in the marker enzyme, alkaline phosphatase in the liver after 96 h of nonylphenol exposure. Alkaline phosphatase serves as diagnostic tool to assess the toxicity stress of chemicals in the living organisms²⁰. Alkaline phosphatase is a hydrolytic lysosomal enzyme and is released by the lysosomes for the hydrolysis of foreign material. Subsequently the enzyme activity may begin to drop either as a result of having partly or fully encountered the

toxin or as a result of cell damage. Alkaline phosphatase is also involved in the mediation of membrane transport and transphosphorylation. A decreased alkaline phosphatase activity in liver of nonylphenol-treated fish for 96 h and the activity of enzyme increased to normal level at 7 days of exposure indicate the decreased state of inter and intracellular membrane transport. This could be possibly due to the toxicity of nonylphenol at acute exposure and the stress is overcome during sub-chronic exposure.

Histopathological observations disclose that the control liver exhibited a normal architecture and there were no pathological abnormalities, with hepatocytes presenting a homogenous cytoplasm and a large central or subcentral spherical nucleus. The hepatic parenchyma of fish exposed to nonylphenol after 7 days showed an increase of cytoplasmic vacuolization. Additionally, hepatocellular necrosis and decrease in the number of hepatocyte nucleus was also observed. During the present investigation the widespread vacuolization might be likely due to accumulation of glycogen in hepatocytes²¹. Hepatocellular lipid vacuolization commonly occurs in fish as a histopathological response to aquatic pollutants. Also, the widespread vacuolization of the liver might be a common response in fish hepatocyte to various chemical stressors, which indicates a higher hepatocellular lipid, water and/ or glycogen content²². Anomalies such as irregular shaped hepatocytes, cytoplasmic vacuolization and nucleus in a lateral position, close to the cell membrane were also described in *Oreochromis mossambicus* when exposed to malathion²³.

V. CONCLUSIONS

It is evident from the present study that the generation of oxygen free radicals induced lipid peroxidation in liver tissues and this might be due to the sub lethal toxicity of nonylphenol in the fresh water fish, *Oreochromis mossambicus*. However, on the basis of statistical observation it is, therefore, understood that the activity of antioxidant enzymes brings to the normal level at 7 days of exposure in the hepatocytes, stating that the liver could have begin to detoxify the exposed toxicant. These data do not warrant a causal connection per se, but taken together with reports in the literature, they strongly suggest a consequence of imbalance in pro-oxidant and antioxidant balance in liver tissues at acute exposure of nonylphenol in the freshwater teleost fish.

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